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Serology and cytokine profiles in patients infected with the newly discovered *Bundibugyo ebolavirus*

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ABSTRACT

A new species of *Ebolavirus*, *Bundibugyo ebolavirus*, was discovered in an outbreak in western Uganda in November 2007. To study the correlation between fatal infection and immune response in *Bundibugyo ebolavirus* infection, viral antigen, antibodies, and 17 soluble factors important for innate immunity were examined in 44 patient samples. Using Luminex assays, we found that fatal infection was associated with high levels of viral antigen, low levels of pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, TNF-α, and high levels of immunosuppressor cytokines like IL-10. Also, acute infected patients died in spite of generating high levels of antibodies against the virus. Thus, our results imply that disease severity in these patients is not due to the multi-organ failure and septic shock caused by a flood of inflammatory cytokines, as seen in infections with other *Ebolavirus* species.

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Introduction

Ebola hemorrhagic fever (EHF) is caused by several species of *Ebolavirus*. Before 2007, 2 of the 4 known *Ebolavirus* species were responsible for human epidemics: *Zaire ebolavirus* and *Sudan ebolavirus*. Between 1976 and 2008, *Zaire ebolavirus* has caused several outbreaks in the Democratic Republic of Congo, Republic of Congo, and Gabon, with fatality rates as high as 90% (Sanchez et al., 2007). *Sudan ebolavirus* has caused multiple outbreaks in Sudan and the Gulu district of Uganda, with approximately 50% case fatality. No reports of human epidemics caused by the other 2 species, *Côte d'Ivoire* and *Reston ebolavirus*es, have been reported. *Côte d'Ivoire ebolavirus* caused a single non-fatal human infection, and *Reston ebolavirus* produces fatal infection in non-human primates (Feldmann and Geisbert, 2011). In November 2007, *Bundibugyo ebolavirus*, a proposed new species of *Ebolavirus*, was discovered in western Uganda. The outbreak began in August of 2007 and ended in December of the same year. In total, 131 cases were identified (MacNeil et al., 2011, JID in press), with 40% case fatality among laboratory-confirmed cases (MacNeil et al., 2010). *Bundibugyo ebolavirus* is most closely related to *Côte d'Ivoire ebolavirus*, sharing approximately 60% gene homology (Towner et al., 2008) and strong IgG antibody cross-reactivity (Macneil et al., 2011). Interestingly, the glycoprotein (GP) differs by 35% at the amino acid level between *Bundibugyo* and *Zaire* viruses, and by 27% between *Bundibugyo and Côte d'Ivoire ebolavirus*es (Towner et al., 2008).

Despite the significant differences in genotype and mortality rates, *Zaire, Sudan*, and *Bundibugyo ebolavirus*es induce a fairly similar clinical manifestation in humans and display the same cell tropism in vitro (Gupta et al., 2010; MacNeil et al., 2010). The viruses replicate primarily in monocytes and dendritic cells, and spread to hepatocytes, endothelial cells, and epithelial cells to cause systemic infection (Sanchez et al., 2007). High viremia induces secretion of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1β (MIP-1β), and interleukins (IL)-1β, 6, and 10. In the absence of substantial host immunity, high viremia leads to septic shock seen in fatal infections, which causes multi-organ failure and death (Feldmann and Geisbert, 2011).

During the course of the *Bundibugyo* EHF outbreak, preliminary laboratory testing was performed in Uganda, and aliquots of diagnostic samples were shipped to the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) for confirmatory testing. We examined circulating levels of cytokines and chemokines, viral antigen Ag, IgM, and IgG levels, in anonymized diagnostic samples from laboratory-confirmed EHF cases (n = 44). Of the 44 samples, 11 were collected during the acute stage of the disease from individuals who died of EHF, 12 were paired samples collected from EHF survivors during the acute and convalescent stages, and 9 were collected from EHF survivors during the convalescent stage. Additionally, as a control, we examined the same serological parameters in samples collected from 10 healthy African health care workers employed at the hospital and involved in outbreak response activities but showed no evidence of the disease. We found that the serum circulating cytokine profiles of

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patients infected with *Bundibugyo ebolavirus* differed significantly from those produced by *Zaire* and *Sudan ebolaviruses*.

Results

Ag, IgM, and IgG levels in acute and convalescent patients infected with Bundibugyo ebolavirus

A total of 44 samples of blood plasma or sera were tested for *Bundibugyo ebolavirus*-specific Ag and antibodies. Samples were divided into acute and convalescent groups. The acute samples were collected 0–11 days after onset of illness from 11 patients who did not survive the infection (acute non-survivors), and 12 surviving patients (acute survivors). No differences were noted in the time between illness onset and sample collection between acute survivors and acute non-survivors (median = 7.5 days among survivors, 7.0 days among non-survivors; $p = 0.6639$). The convalescent group included 21 samples collected during the convalescent stage of the disease, 35–64 days after illness onset; 12 of these samples were collected from the same patients used for the acute survivors group, while the remaining 9 did not have a paired acute sample. Ag titers ranged from 150 to 12,150 among acute non-survivors (median = 4050; Fig. 1a; Antigen titers are dilutions corrected at $OD_{410} > 0.1$); 6 of the 11 acute non-survivors had titers of 4050. Ag titers were significantly lower (50–4050; median = 150) in the acute survivors group, and 3 of the samples did not contain detectable Ag. As expected, no Ag was detected in convalescent patients (Fig. 1a).

We determined the levels of anti-*Bundibugyo ebolavirus* IgM and IgG antibodies using capture ELISA. Four of the 11 non-survivors had IgM titers of 1:2560, 4 had no detectable antibodies, and 3 had titers ranging from 1:40 to 160. IgG levels in these patients ranged from 1:40 to 2560; 3 of the 11 samples had IgG titers of 1:2560, and 4 had titers of 1:640. Two samples did not have any detectable anti-Ebola IgG (Fig. 1b).

Anti-*Ebolavirus* IgM levels were similar in acute survivors, ranging from 1:160 to 2560. Five of the 12 patients had titers of 1:640 and 3 had titers of 1:2560; no IgM was detected in 3 samples. Anti-Ebola IgG levels in these patients were also similar to those of acute non-survivors (1:160–2560). Three of the 12 patients had titers of 1:2560, 4 had titers of 1:640, and 4 had titers of 1:160. Patients with high levels of IgM also had corresponding levels of IgG; for example, 3 patients had titers of 1:640–2560 of both IgM and IgG (Figs. 1b, c).

Convalescent patients expressed anti-*Ebolavirus* IgM levels 35–64 days post-infection. Twelve of the 21 patients had IgM levels ranging 1:40–640, and 1 sample reached 1:2560. All convalescent patients also expressed anti-*Ebolavirus* IgG. Seven patients had titers of 1:2560, and 8 had titers of 1:10,240 (Figs. 1b, c). Samples from the 10 individuals with no disease were serologically negative for both anti-*Bundibugyo Ebolavirus* IgM and IgG and negative for *Ebolavirus* antigen (anti-*Bundibugyo ebolavirus* IgM and IgG titer <40 and *Ebolavirus* antigen titer <50). A table of contents summarizes laboratory results of viral antigen, anti-*Bundibugyo ebolavirus* IgM and IgG antibodies, time of sample collection from the onset of illness and outcome of the disease on all 44 samples collected is shown (Table 1).

Reduced IL-1α, IL-1β, IL-6, and TNF-α levels, and increased IL-10 levels in acute samples

In order to identify the cytokines affected by *Bundibugyo ebolavirus* infection, we compared cytokine levels in acute-stage and convalescent-stage samples. We found that several pro-inflammatory cytokines were not induced during acute disease. IL-1α and IL-1β were significantly lower in acute patients than in convalescent patients ($p < 0.005$). Indeed, IL-1α was undetectable in 23 of the 24 acute patients, and IL1β was undetectable in 20 of the 24

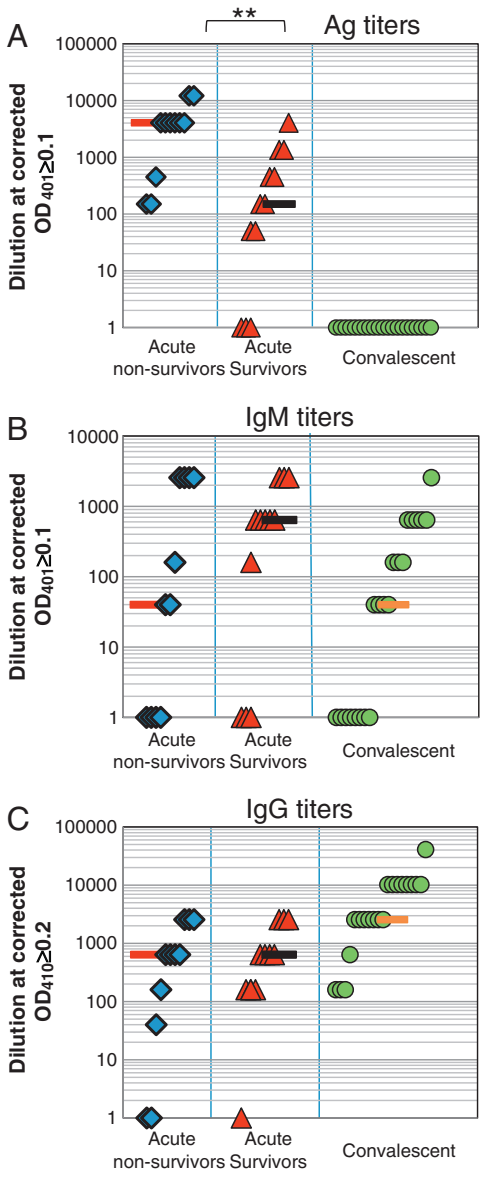

Fig. 1. Antigen (Ag), IgM, and IgG levels in patients infected with *Bundibugyo ebolavirus*. Antigen (A), anti-*Ebolavirus* IgM (B), and anti-*Ebolavirus* IgG (C) levels were determined by immunocapture or direct enzyme-linked immunosorbent assay (ELISA) in samples collected from non-surviving (diamonds; n = 11) and surviving patients (triangles; n = 12) during the acute stage of *Bundibugyo ebolavirus* infection, and in samples from convalescent patients (circles; n = 21). Each point represents 1 sample. Median values are shown as dashed lines. Viral antigen levels were significantly higher in acute samples from non-survivors than survivors (**$p < 0.005$). We saw no differences in anti-*Ebolavirus* IgM and IgG levels between non-surviving and surviving patients.

patients (Fig. 2A). Similarly, inflammatory cytokine levels were also significantly lower in acute than in convalescent patients ($p < 0.005$). Median TNF-α levels were 4-fold lower in acute than convalescent patients (10 pg/mL vs 42.7 pg/mL, respectively; Fig. 2B), while median IL-6 levels were 2 logs lower in acute patients than in convalescent patients (15 pg/mL vs 299 pg/mL, respectively; Fig. 2A). Interestingly, we found that the levels of the anti-inflammatory immunosuppressor IL-10 were approximately 20-fold higher in the acute group than in the convalescent group (144 pg/mL vs 6 pg/mL, respectively; Fig. 2B). Together, these data suggest that patients generally express low levels of inflammatory cytokines during the acute stage of Bundibugyo EHF.

We found no significant differences in IFN-γ levels between samples from controls and acute patients or between acute and convalescent patients. We found that IFN-α2 levels were significantly higher in acute patients than in convalescent patients ($p = 0.02$), but not

Table 1
Summary of laboratory results of viral antigen, anti-*Bundibugyo Ebolavirus* IgM and IgG antibodies, diagnostic PCR results (done in the field), sample collection time from the onset of illness and outcome of disease on all 44 samples collected during outbreak. ND means not determined.

Sample type	Case ID	Diagnostic PCR	Collection time	Ag	IgM	IgG	Outcome
Unpaired, acute	603	Positive	11	4050	40	<40	Dead
Unpaired, acute	609	Positive	6	4050	2560	2560	Dead
Unpaired, acute	610	Positive	8	12,150	2560	2560	Dead
Unpaired, acute	614	Positive	9	4050	<40	2560	Dead
Unpaired, acute	627	Positive	4	450	<40	640	Dead
Unpaired, acute	630	Positive	0	150	<40	640	Dead
Unpaired, acute	631	Positive	5	4050	160	160	Dead
Unpaired, acute	633	Positive	9	12,150	2560	640	Dead
Unpaired, acute	634	Positive	Unknown	4050	<40	640	Dead
Unpaired, acute	636	Positive	7	4050	2560	<40	Dead
Unpaired, acute	638	Positive	7	150	40	40	Dead
Paired	605	ND	2	<50	<40	160	Alive
	605		42	<50	<40	160	Alive
Paired	611	Positive	9	450	2560	160	Alive
	611		47	<50	160	2560	Alive
Paired	612	Positive	9	450	2560	640	Alive
	612		53	<50	640	2560	Alive
Paired	613	Positive	7	1350	640	160	Alive
	613		48	<50	640	10,240	Alive
Paired	616	Positive	10	150	640	640	Alive
	616		55	<50	<40	10,240	Alive
Paired	617	Positive	9	50	640	640	Alive
	617		46	<50	640	10,240	Alive
Paired	619	Positive	5	<50	<40	640	Alive
	619		44	<50	<40	160	Alive
Paired	621	Positive	8	4050	160	2560	Alive
	621		47	<50	40	2560	Alive
Paired	623	Positive	5	50	<40	160	Alive
	623		44	<50	<40	10,240	Alive
Paired	626	Positive	7	150	640	ND	Alive
	626		36	<50	40	10,240	Alive
Paired	628	Positive	7	<50	640	2560	Alive
	628		47	<50	40	2560	Alive
Paired	635	Positive	8	1350	2560	2560	Alive
	635		35	<50	640	2560	Alive
Unpaired, convalescent	602	ND	62	<50	<40	10,240	Alive
Unpaired, convalescent	604	ND	45	<50	<40	160	Alive
Unpaired, convalescent	607	ND	45	<50	160	ND	Alive
Unpaired, convalescent	608	ND	46	<50	640	2560	Alive
Unpaired, convalescent	622	ND	40	<50	40	10,240	Alive
Unpaired, convalescent	624	ND	47	<50	2560	2560	Alive
Unpaired, convalescent	629	ND	62	<50	<40	40,960	Alive
Unpaired, convalescent	632	ND	64	<50	<40	640	Alive
Unpaired, convalescent	637	ND	46	<50	160	10,240	Alive

higher than in controls. MCP-1 levels were significantly higher in acute patients and convalescent patients than in controls (Fig. 2C). Interestingly, in convalescent patients, MCP-1 levels were even significantly higher than in acute patients (median = 4637 pg/mL vs 2387 pg/mL; $p<0.05$; Fig. 2C).

To further examine these trends, we compared cytokine levels in acute or convalescents samples with the samples from the 10 individuals with no evidence of disease. As we previously observed, IL-1α, IL-1β, TNF-α, and IL-6 were lower, and IL-10 higher, in acute samples compared to samples from control individuals. Furthermore, the levels of these cytokines did not differ between the control samples and convalescent samples.

IFN-α2, IFN-γ, and IL-10 levels are higher in acute non-survivors than in acute survivors

In order to identify cytokines associated with disease severity, we specifically examined cytokine levels in samples collected during the acute stage of the disease, and stratified these values according to patient survival. IFN-α2 levels were significantly higher in non-survivors than in survivors (median = 45 pg/mL vs 1 pg/mL, respectively; $p<0.05$). Ten of the 12 survivors had no detectable IFN-α2 levels. Similarly, IFN-γ levels were significantly higher in non-survivors than in survivors (median = 30 pg/mL vs 9 pg/mL, respectively). Interestingly, our results show that IL-10 levels were 3-fold higher in non-survivors than in survivors (median = 597 pg/mL vs 169 pg/mL, respectively; $p<0.005$; Fig. 3). We did not observe significant differences in IL-6, MCP-1, TNF-α, IL-1α, or IL-1β levels between these 2 groups.

Discussion

The *Bundibugyo ebolavirus* outbreak produced 131 reported cases with an approximate mortality rate of 40%. We examined 11 samples collected during the acute stage of the disease from non-survivors, and 12 collected from survivors, as well as 21 samples collected from convalescent patients. Our data show that median Ag titers were about 25-fold higher in acute non-survivors than in acute survivors (150 vs 4050). Interestingly, individuals who had high viral Ag titers had correspondingly high IgG titers (data not shown). Elevated IgG levels in non-survivors were rarely observed in previous outbreaks (Baize et al., 1999, 2002; Ksiazek et al., 1999), and suggest that patients infected with *Bundibugyo ebolavirus* may develop higher cellular and humoral immunity than do patients infected with other *Ebolavirus* species. We have previously shown that monocyte cell death is delayed in *Bundibugyo ebolavirus* infection compared to *Zaire ebolavirus* infection in vitro (Gupta et al., 2010). It is possible

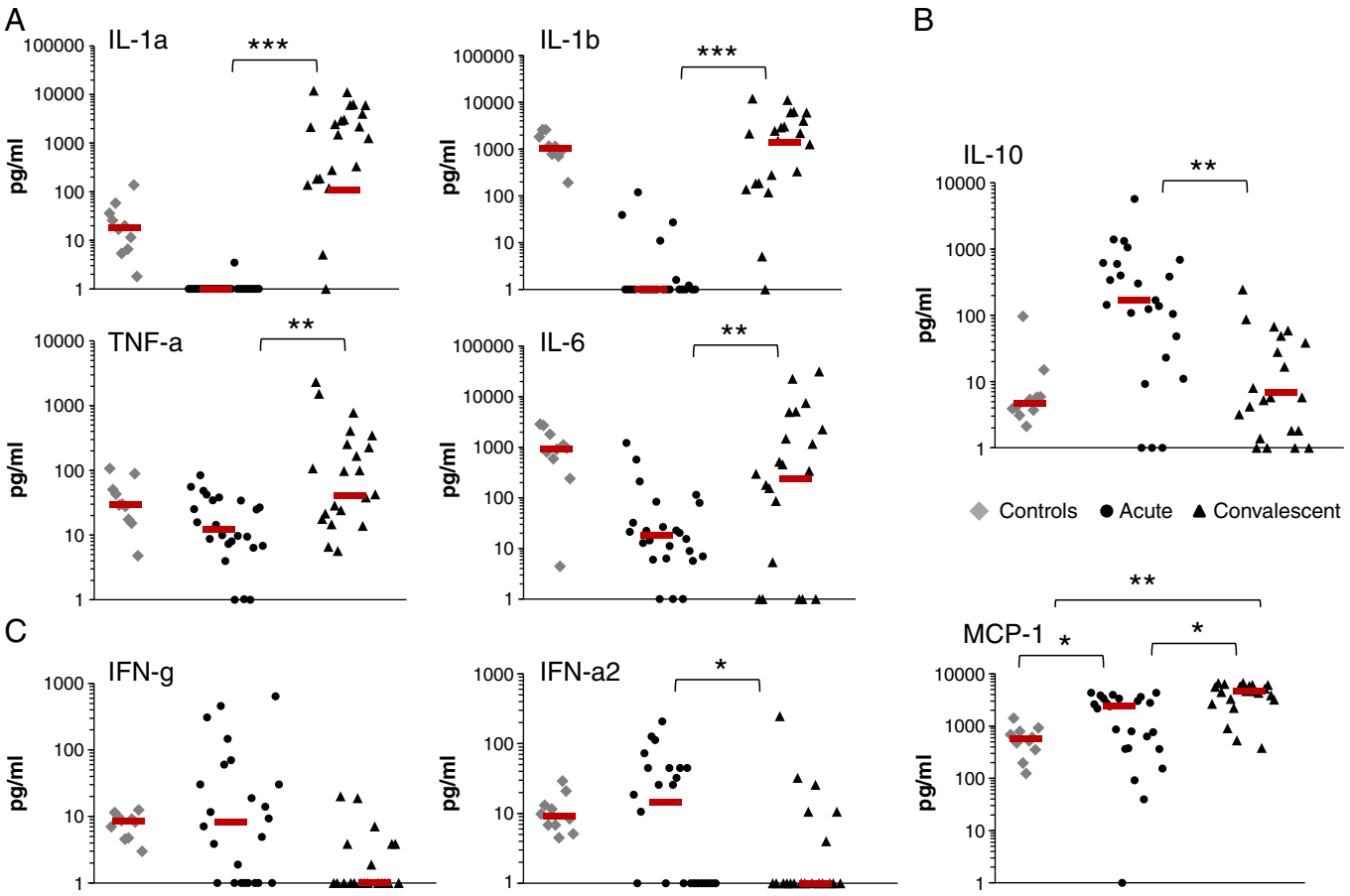

Fig. 2. Cytokine and chemokine profiles in samples from control, acute-stage, and convalescent patients. A. IL-1α, IL-1β, TNF-α, and IL-6 levels. B. IL-10 levels. C. IFN-γ, IFN-α2, and MCP-1 levels. Each dot represents 1 sample, and dashes (—) represent median values. *Indicates $p<0.05$, **indicates $p<0.005$, ***indicates $p<0.0005$.

that this delay in monocyte and/or lymphocyte death in *Bundibugyo ebolavirus*-infected patients activates humoral immunity. In spite of the anti-viral IgM and IgG response in non-survivors, these patients were unable to control infection and succumbed to it. However it is possible that antibodies generated during infection in non-survivors are simply a reflection of viral load and are not necessarily controlling viral infection.

We observed significantly lower IL-1α, IL-1β, IL-6, and TNF-α levels in acute samples than in convalescent or control samples, suggesting that these cytokines are downregulated by *Bundibugyo ebolavirus*. IL-1α possesses hemopoietic activity (Arend, 2002) and induces CD4 T-cell and B-cell proliferation, as well as TNF-α release by endothelial cells (Smirnova et al., 2002). IL-1α synthesis is inhibited by IL-10 (Smirnova et al., 2002), so it is possible that viral replication may indirectly inhibit IL-1α, probably by inducing IL-10 (see below). Also, we did not detect IL-1β, another important regulator of the inflammatory response, in samples from acute patients. Likewise, non-surviving patient infected with *Zaire ebolavirus* in Gabon had no detectable IL-1β levels (Baize et al., 2002). We found significantly lower IL-6 and TNF-α levels in acute samples than in controls and convalescent samples. In addition to previous studies, which report elevated IL-6 and TNF-α levels in samples from non-surviving patients, we found no differences in the levels of these cytokines between the acute survivor and non-survivor groups (Hutchinson and Rollin, 2007; Wauquier et al., 2010). This suggests that, unlike other *Ebolavirus* species, *Bundibugyo ebolavirus* does not induce a strong inflammatory response.

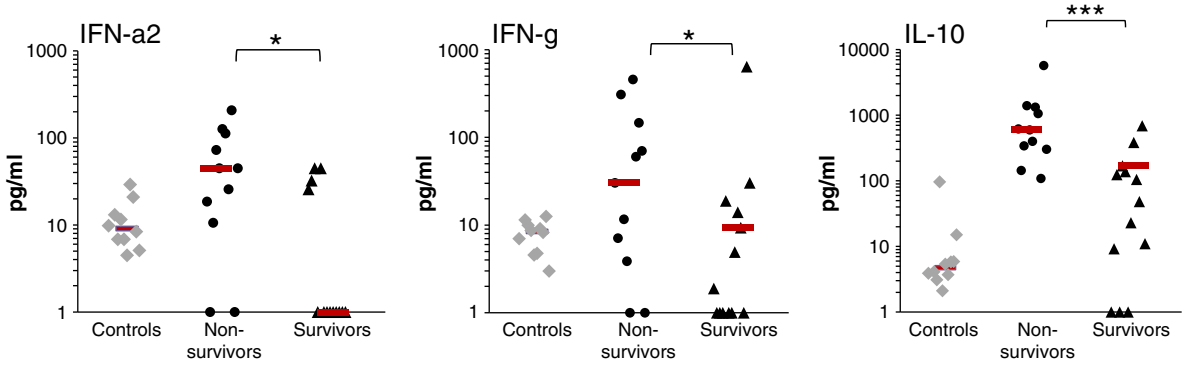

Fig. 3. IFN-α, IFN-γ, and IL-10 levels in acute-stage surviving patients (n = 12) or non-surviving patients (n = 11). Data are plotted as pg/mL. Each symbol represents 1 sample. *Indicates $p<0.05$, **indicates $p<0.005$, and ***indicates $p<0.0005$.

Consistent with previous findings, we found that non-surviving patients had significantly higher levels of IL-10 than did survivors (Baize et al., 2002; Hutchinson and Rollin, 2007; Villinger et al., 1999). IL-10 is an anti-inflammatory immunosuppressor cytokine primarily produced by the monocytes. It downregulates Th1 response and inhibits CD8 T-cell response (O'Garra et al., 2008), and suppresses IFN-γ, IL-2, and TNF-α, thus inhibiting the inflammatory response (Brockman et al., 2009). Recently, IL-10 has been found to block nitric oxide synthesis in monocytes, affecting the anti-viral function of these cells (Burgner et al., 1999; Moreira et al., 2010). High levels of IL-10 have been observed in acute-stage patients infected with other *Ebolavirus* species, suggesting that the *Ebolavirus* motif that induces IL-10 secretion has been evolutionarily conserved, and has continued to suppress host immunity. Therefore, we think that high IL-10 levels detected a few days after illness onset may be potential marker of fatal outcome. Possibly *Ebolavirus* infected monocytes and dendritic cells are the main source of IL-10 induction. Studies are underway to confirm this hypothesis.

The release of pro-inflammatory cytokines and chemokines has been proposed to cause fatality in *Ebolavirus* infection (Feldmann and Geisbert, 2011), and previous studies report high levels of IFN-γ, IFN-α, and TNF-α in cases of fatal *Ebolavirus* infection (Baize et al., 1999; Villinger et al., 1999). Interestingly, although we also found higher levels of IFN-γ and IFN-α in non-survivors than in survivors, but did not see significant differences in TNF-α levels between the 2 groups. A prominent feature of *Bundibugyo ebolavirus*, therefore, is downregulation of inflammatory cytokines and upregulation of immunosuppressor cytokines. Our study suggests that high levels of inflammatory cytokines, which can lead to septic shock, do not seem to cause death in *Bundibugyo ebolavirus* infection.

A previous study performed in asymptomatic patients reported that individuals seropositive for *Ebolavirus* express high levels of inflammatory cytokines (Leroy et al., 2000). Our results show a similar trend.

In conclusion, we have shown that pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-1α, and IL-6 are not induced in non-surviving patients, suggesting that death in these patients is not caused by septic shock from a cytokine flood, as previously suggested for other species of *Ebolavirus*. However, IL-10 was higher in non-survivors than in survivors, as observed previously in outbreaks of both *Zaire* and *Sudan ebolavirus* species (Hutchinson and Rollin, 2007; Villinger et al., 1999). Studies to investigate the role of IL-10 in host immunity against different species of *Ebolavirus* are currently underway.

Materials and methods

Samples

During outbreak response, aliquots of diagnostic samples were shipped to the CDC for confirmatory testing. All samples were collected as part of outbreak activities, anonymized, and identified only as fatal and nonfatal cases. Human subject review of this protocol was performed and approved by the CDC Institutional Review Board. Upon collection, samples were taken to Biosafety level-4 (BSL-4) and stored at −80 °C until further processing. Plasma and serum were separated by centrifugation and stored in liquid nitrogen. All samples were inactivated by gamma-irradiation (5×10^6 rads) before determining Ag, antibody, cytokine, and chemokine levels in a BSL-2 laboratory.

Viral Ag assay

Levels of circulating *Ebolavirus* Ag in plasma and sera were estimated using a capture enzyme-linked immunosorbent assay (ELISA) as previously described (Ksiazek et al., 1999). Briefly, Flexiplates (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) were coated overnight at 4 °C with mouse ascitic fluid containing a mixture of 7 mouse monoclonal antibodies raised against viral protein VP40, GP, and nucleoprotein (NP) derived from Zaire 1976 and Sudan 1976 samples of *Ebolavirus*. Plasma and serum samples were serially diluted in 5% skim milk in phosphate-buffered saline (PBS; pH 7.2) with 0.1% Tween-20 (hereafter referred to as blocking buffer), and incubated at 37 °C for 1 h. Captured Ag was detected using polyclonal anti-*Zaire ebolavirus* serum produced in rabbits, and visualized with a goat anti-rabbit horseradish peroxidase (HRP) conjugate and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium (ABTS) substrate (all from Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical density at 410 nm (OD_{410}) of each sample placed into control wells coated with normal mouse ascitic fluid was subtracted from the OD of the same sample placed into the corresponding well coated with anti-*Ebolavirus* monoclonal antibodies to derive a corrected OD. The highest sample dilution that resulted in a corrected OD ≥0.1 was designated as the viral Ag titer of the sample.

Anti-Ebolavirus IgG detection assay

Levels of circulating anti-*Bundibugyo ebolavirus*-specific antibodies were detected using capture ELISA as previously described (Ksiazek et al., 1999). Flexiplates were coated overnight at 4 °C with lysates of Vero E6 infected with *Bundibugyo ebolavirus* or with uninfected Vero E6 cell lysate. Plasma and serum samples were serially diluted in skim milk as described above. Capture antibodies were detected using mouse anti-human IgG-HRP conjugate and visualized with ABTS substrate as above. The highest sample dilution that resulted in a corrected OD≥0.2 was designated as the antiviral IgG titer of the sample.

Anti-Ebolavirus IgM assay

Anti-*Ebolavirus* IgM antibodies were detected by sandwich ELISA as previously described (Ksiazek et al., 1999). Flexiplates were coated with anti-mouse antibodies (Biosource International, Camarillo, CA, USA) overnight at 4 °C. Serially diluted samples were prepared in skim milk as described above and incubated for 1 h at 37 °C. To estimate the amount of anti-*Ebolavirus*-specific antibodies, samples were incubated with *Bundibugyo ebolavirus*-specific cell slurry or with normal cell slurry for 1 h at 37 °C. *Ebolavirus*-specific antibodies were detected using polyclonal anti-*Ebolavirus* antibodies and goat anti-mouse-HRP conjugate. Color was developed with ABTS substrate as described above. The highest dilution of sample that resulted in a corrected OD ≥0.1 was designated as the anti-viral IgM titer of the sample.

Cytokine detection using Luminex beads

Levels of 17 cytokines and chemokines were detected using Luminex technology (Bio-Rad Laboratories, Hercules, CA, USA). One customized cytokine kit was used according to manufacturer's instructions. The target cytokines were IL-1α, IL-1β, IL-10, IL-6, IL-12p40, IL-12p70, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, interferon α2 (IFN-α2), IFN-γ, IFN-inducible protein (IP-10), T-cell growth factor α (TGF-α), regulated-on-activation, normal T-cell expressed, and secreted (RANTES), and vascular cell adhesion molecule-1 (VCAM-1).

After inactivation by irradiation, the samples were processed under BSL-2 conditions; previous studies show that the irradiation process does not affect the cytokine assay (Mahanty et al., 1999). Briefly, 25 μL of plasma or serum were diluted 1:4 and incubated with antibody-coupled beads for 1 h at room temperature. The beads were then incubated at room temperature with detector antibody for 1 h, and with streptavidin-phycoerythrin for 30 min. Finally, the complexes were washed and resuspended in running buffer. The

beads were washed 3 times between each step according to manufacturer's instructions. 100 beads per cytokine were acquired using Luminex 400 (Luminex Corp., Austin, TX, USA). Mean fluorescent intensity was measured to calculate final cytokine concentration in pg/mL.

Statistical analysis

Statistical analyses were performed using standardized software (SAS v9.2). Based on a reliable detection limit of 1 pg/mL, individually measured cytokine values below this value were designated as 1 pg/mL for analytic purposes. Differences in cytokine values between study groups were assessed by Wilcoxon rank sum test; p-values represent 2-sided p-values, and p values <0.05 were considered statistically significant.

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